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Single Oral Immunization with Replication Deficient Recombinant Adenovirus Elicits Long-Lived Transgene-Specific Cellular and Humoral Immune Responses

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Oral–gastric delivery of vaccines is a preferred route of immunization and is particularly relevant to the development of vaccine-vector systems. We have investigated the ability of a replication deficient (E1-deleted) adenovirus construct (RAd68), which efficiently expresses the measles virus nucleocapsid (N) protein under the control of the strong HCMV IE promoter, to elicit antibody and cytotoxic T cell (CTL) responses in mice following intragastric administration. Measles virus N protein-specific CTL memory and serum antibody responses were analyzed in a total of 140 mice at time points 2–51 weeks after immunization either with a single dose of 10^8 pfu RAd68 or with a fivefold higher dose. Of the 20 animals analyzed in the first 4-week period following low-dose immunization, 6 mounted low-level splenic CTL responses while 13 animals had CTL in the mesenteric lymph nodes. Splenic CTL responses were largely undetectable at later times. Only 23% of low-dose-immunized mice made serum antibody responses and these were generally of low magnitude and frequently of short duration. In contrast, the majority of animals immunized orally with 5×10^8 pfu RAd68 mounted splenic CTL responses (70%) and/or antibody responses (89%). Notably, these responses were stronger and of greater duration than those seen following immunization at the lower dose. Gut mucosal immunization with replication deficient adenoviruses is a promising approach, not only for the development of complementary measles vaccine strategies which may be required for measles virus eradication, but also generally for vaccination against other infections.

Key Words: oral immunization; replication deficient adenovirus; antibody; CTL.

INTRODUCTION

The properties of adenovirus vectors have recently been extensively reviewed (Carroll *et al.,* 2001; Russell, 2000). As well as being able to express high levels of transgene products, these vectors are particularly attractive since they can stimulate long-term humoral and cellular immune responses directed to the expressed product (Juillard *et al.,* 1995). Importantly, both systemic and local immune responses can be induced with such vectors (Gallichan and Rosenthal, 1996; Xiang and Ertl, 1999). Oral administration of live adenovirus has been used successfully for many years in the United States as a vaccine strategy for the prevention of acute respiratory disease in military recruits (Rubin and Rorke, 1994). The Ad4 and Ad7 strains associated with respiratory tract infection were found to replicate in the gut in the absence of disease induction, thus removing the need for specific attenuation (Chanock *et al.,* 1966). However, oral immunization with recombinant adenoviruses to induce transgene-specific responses has received surprisingly little attention. This strategy has been employed mainly with replication competent viruses where the expression of the transgene is driven by an endogenous adenovirus promoter (Lubeck *et al.,* 1989; Prevec *et al.,* 1990; Both *et al.,* 1993; Flanagan *et al.,* 1997).

Replication deficient vectors have distinct advantages for immunization including enhanced safety and containment properties. This may be particularly relevant for use in populations that may contain immunocompromised individuals. In addition, because cell to cell spread is not a requirement, such vectors may be less influenced by preexisting immunity and, indeed, are less likely to stimulate strong anti-vector immunity themselves.

In this report we have examined the ability of an $E1^-$ replication defective human adenovirus 5 recombinant expressing the measles virus nucleocapsid protein (MV N) under the control of the HCMV major IE promoter (Fooks *et al.,* 1995) to induce persistent humoral and memory cytotoxic T lymphocyte (CTL) responses in mice following oral administration at two different doses. We selected the measles virus nucleocapsid protein as a model antigen for this study as our laboratory has a long-standing interest in the development of complementary measles virus vaccine strategies (Fooks *et al.,* 2000).

RESULTS

MV N protein-specific CTL responses in mice immunized with 10^8 pfu RAd68

Following oral immunization of a total of 70 mice each with 10^8 pfu of recombinant adenovirus expressing mea-

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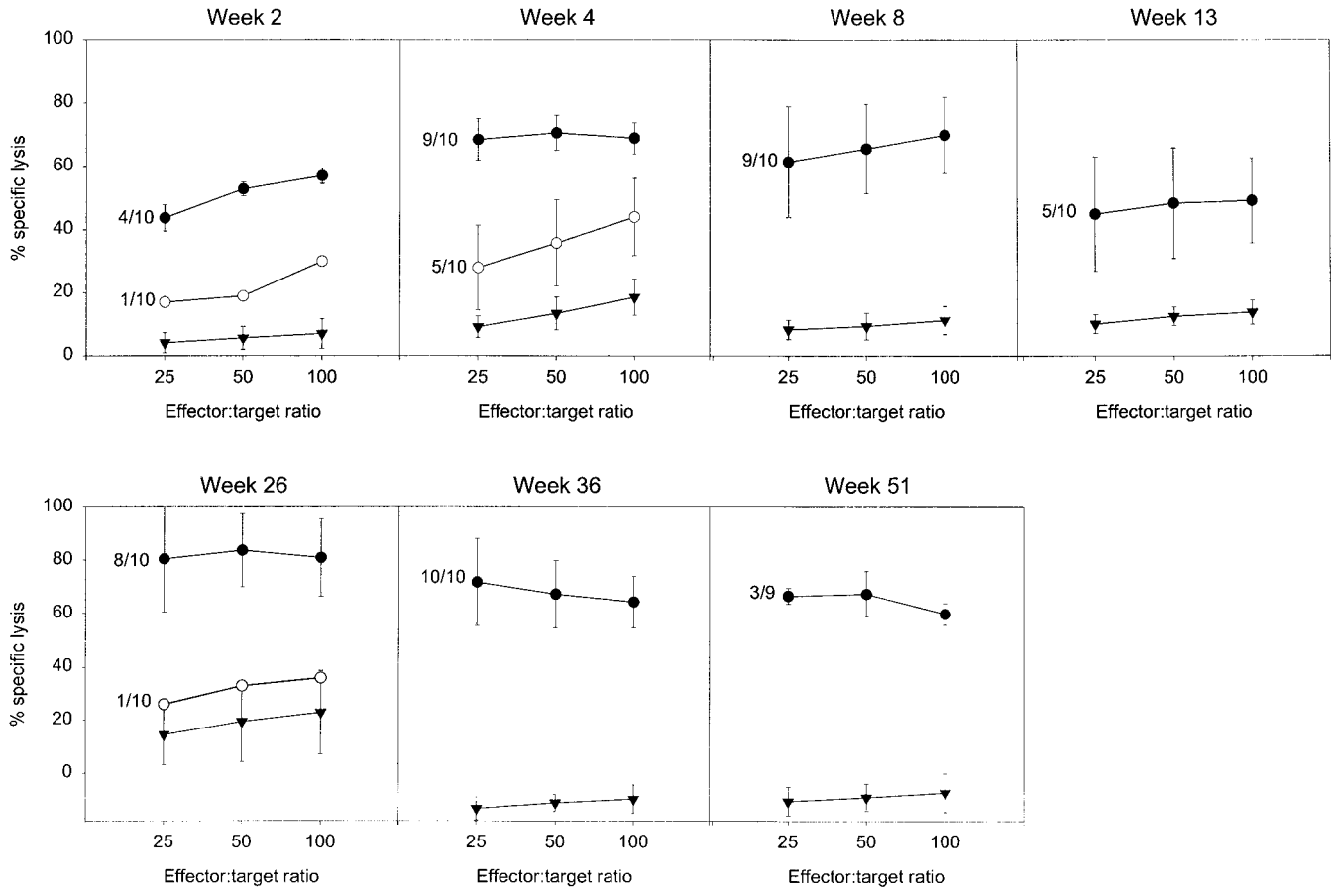

FIG. 1. Measles virus N protein-specific splenic CTL activity following oral immunization with RAd68. Mean ± standard deviation percentage specific lysis of measles virus NP9 peptide-pulsed autologous target cells in responding mice is shown following immunization with a single dose of either 10^8 pfu RAd68 (○) or 5×10^8 pfu Rad68 (●). Specific precursors were expanded *in vitro* by incubation with NP9 peptide. The means were calculated from the positively responding animals only. Responses were assigned as positive where they exceeded the mean background level of killing determined in splenocytes from 18 naive mice by two standard deviations. The proportion of mice responding at each time is shown. ▼, Background killing of autologous targets in the absence of peptide.

sles virus nucleoprotein (RAd68), the longevity of any CTL responses was analyzed in groups of 10 mice on 7 occasions up to 51 weeks. After restimulation of effector cells *in vitro* with the N-protein CTL epitope, NP9, splenic responses were detected in only 7 of 70 mice analyzed. These responses were low level and, with the exception of 1 animal, seen only during the first month (Fig. 1). In contrast, 7 of 10 mice examined at week 2 and 6 of 8 (no cells recovered from 2 mice) examined at week 4 had CTL responses in the mesenteric lymph nodes (Fig. 2). Of the 13 animals with mesenteric CTL, only 2 (at week 4) had splenic responses. At week 8, insufficient viable cells were recovered for analysis after restimulation of mesenteric lymphocytes with peptide.

MV N protein-specific CTL responses in mice immunized with 5×10^8 pfu RAd68

Oral immunization with the higher dose of RAd68 induced splenic responses in 48 of 69 mice tested (1 mouse died during the study of causes unrelated to immunization). Furthermore, responses were higher than those seen following low-dose immunization (Fig. 1). By week 4, the majority of animals had responded and the mean lytic activity had increased from that seen at week 2. Mean responses remained high throughout the course of the experiment, with the exception of results obtained at week 13 (when fewer mice responded). On this occasion, however, only 1 of 2 control mice, inoculated intraperitoneally, responded, whereas on every other occasion both the positive controls had CTL activity. By week 51 the frequency of splenic CTL responsiveness was reduced, although the magnitude of the response had not diminished.

In contrast to mice inoculated orally at the lower dose, only 4 of 10 animals examined at week 2 had mesenteric lymph node CTL responses, which, in general, were weaker than those responses seen in the low-dose group. Two animals had mesenteric CTL in the absence of splenic activity. By week 4 mesenteric responses were undetectable in 5 mice from which viable mesenteric

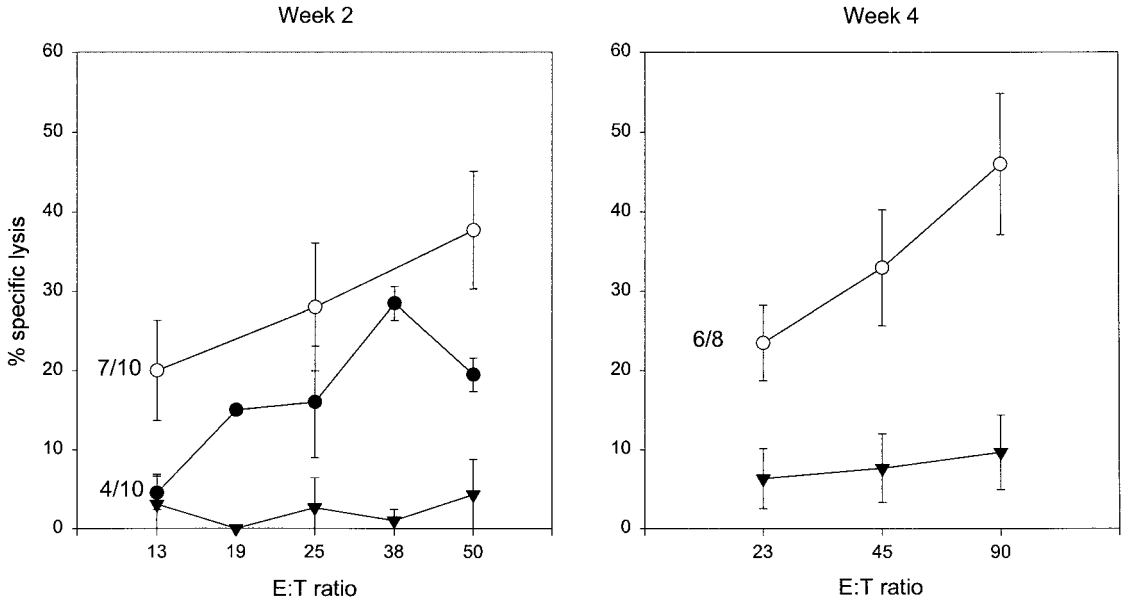

FIG. 2. Measles virus N protein-specific CTL activity in mesenteric lymphocytes early after oral administration of RAd68. Mean ± standard deviation percentage specific lysis of measles virus NP9 peptide-pulsed autologous target cells in responding mice is shown following immunization with a single dose of either 10^8 pfu RAd68 (○) or 5×10^8 pfu Rad68 (●). The means were calculated from the positively responding animals only. Responses were assigned as positive where they exceeded the mean level of killing determined in mesenteric lymph nodes from six naive mice by two standard deviations. The proportion of mice responding at each time is shown. ▼, Background killing of autologous targets in the absence of peptide.

lymphocytes were recovered following culture, although all 5 had splenic responses. As in the low-dose group, no viable mesenteric lymphocytes were recovered at week 8 following restimulation *in vitro*.

In all experiments, lymphocytes from naive mice were unreactive in the CTL assay.

MV N protein-specific serum antibody responses in mice immunized with 10^8 pfu RAd68

Measles virus N protein-specific antibody responses were detected in 16 of 70 mice. In general, antibody responses were weak (Fig. 3). Of the 7 animals positive

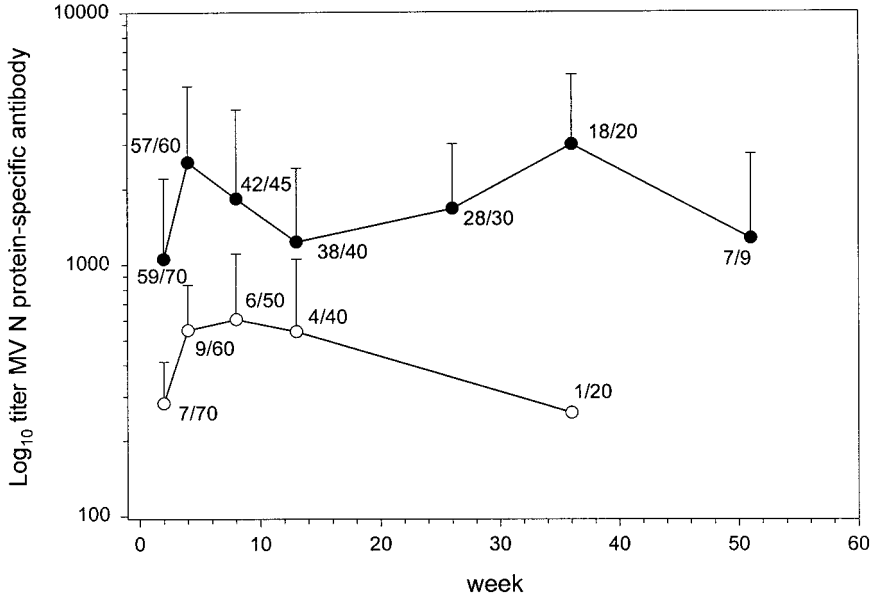

FIG. 3. Longitudinal analysis of measles virus N protein-specific serum antibody titers following oral immunization with RAd68. IgG responses specific for measles virus N protein were measured by ELISA. Mean end point titers + one standard deviation are shown following immunization with a single dose of either 10^8 pfu RAd68 (○) or 5×10^8 pfu RAd68 (●). Means were calculated from the positively responding animals only. Responses were assigned as positive where titers exceeded 100. The proportion of mice responding at each time point is indicated.

TABLE 1

Distribution of Measles Virus N Protein-Specific CTL and Antibody Responses in Individual Mice

	Number of mice responding									
	Low dose[a]					High dose[b]				
Week	CTL[c]/ Ab[d]	CTL/ no Ab	No CTL/ Ab	No CTL/ no Ab	Number tested	CTL/Ab	CTL/ no Ab	No CTL/ Ab	No CTL/ no Ab	Number tested
2	2	6	0	2	10	5	1	0	4	10
4	7	2	0	1	10	9	0	1	0	10
8	0	0	3	7	10	9	0	0	1	10
13	0	0	2	8	10	5	0	5	0	10
26	0	1	0	9	10	8	0	2	0	10
36	0	0	2	8	10	10	0	0	0	10
51	0	0	1	9	10	3	0	4	2	9

[a] Animals immunized with 10^8 pfu RAd68.
[b] Animals immunized with 5×10^8 pfu RAd68.
[c] Indicates a positive response detected in spleen and/or mesenteric lymph nodes.
[d] Indicates antibody response detected at any time after immunization.

at week 2, 3 had titers below the threshold of detection at week 4; another animal had a reduced titer and in only 2 animals was a rising titer detected. Six animals in which antibody was not detected at week 2 became seropositive by week 4 (titers 162–1071). Another 3 animals responded between weeks 4 and 8. Antibody responses were, in general, transient: by week 13, all but 1 responder had falling titers.

The overall pattern of antibody and CTL responses is summarized in Table 1. All animals that had splenic CTL responses made persistent antibody responses, with one exception. In this animal, which never made an antibody response, a CTL response was detected at week 26. Of the 11 animals at weeks 2 and 4 in which CTL responses were detected in the mesenteric lymph nodes but not in the spleen, only 3 had weak antibody responses. In 2 of these animals sacrificed at week 4, antibodies were detected only at week 2. A further 7 animals, sacrificed from week 8 onward for CTL analysis, had antibody responses in the absence of splenic CTL responses; although in only 4 instances were MV-N-specific antibodies detectable at the time of CTL analysis. Of the 3 CTL nonresponders with antibody at week 8, only 1 had previous evidence of seroconversion.

MV N protein-specific serum antibody responses in mice immunized with 5×10^8 pfu RAd68

Measles virus N protein-specific antibody responses were detected in 62 of 70 mice. In general, in contrast to responses in animals given 10^8 pfu, responses were high (Fig. 3). Three mice that were seronegative at week 2 had seroconverted by week 4. Of the 8 animals in which antibody was never detected, 5 were killed at week 2 (for CTL analysis). One other seronegative animal was killed at week 8 and the final 2 animals were unresponsive for the whole period of the experiment.

All CTL responders, including 2 animals at week 2 that only had CTL responses detectable in mesenteric lymph nodes, made antibody responses with the exception of 1 animal analyzed at week 2 (that had both a splenic and a mesenteric lymph node CTL response). Thirteen CTL nonresponders had antibody responses. The mean antibody titers measured over time were lower in this group of animals compared to the titers in animals in which a CTL response was elicited but this difference did not reach statistical significance.

Antibody titers from unvaccinated mice fell below the threshold of detection at all time points.

DISCUSSION

Previous studies of oral immunization with recombinant adenoviruses have largely focused on replication competent constructs. Oral (intragastric) immunization of mice with $E3^-$ recombinant adenovirus expressing rotavirus VP7 induced serum antibody responses, but these were at a lower level than those seen following intraperitoneal, intravenous, or intranasal delivery (Both *et al.,* 1993). Similarly, an $E3^-$ construct expressing rabies virus glycoprotein induced neutralizing antibodies in mice following oral administration, but only a proportion of mice responded and the titers were low compared with those in an intraperitoneally inoculated group (Prevec *et al.,* 1990). In another murine study, an $E3^-$ construct expressing simian immunodeficiency virus Gag p55 induced antibody in fewer than half of the mice immunized; CTL responses were also detected and in some animals were detected in the absence of a serum antibody response (Flanagan *et al.,* 1997). In chimpanzees, a spe-

cies fully permissive for the replication of human adenoviruses, sequential oral administration of $E3^-$ constructs expressing hepatitis B surface antigen induced low levels of antibody (Lubeck *et al.,* 1989). However, in a small human clinical trial, oral administration of one of these constructs failed to induce transgene-specific immunity (Tacket *et al.,* 1992). Overall, replication-competent adenoviruses appear to require a degree of active replication in the vaccinated host in order to stimulate effective transgene-specific immune response (Lubeck *et al.,* 1997; Buge *et al.,* 1997). In the present study, an $E1^-$ replication deficient construct was used that is capable of high level expression of MV N protein driven by the strong HCMV major IE promoter. Previous studies have shown this construct to elicit transgene-specific humoral and CTL immunity in mice following parenteral vaccination (Fooks *et al.,* 1995). A striking finding following oral delivery of this construct was the influence of dose on the responses obtained. Systemic humoral and CTL responses were weaker, of short duration, and of much lower frequency following administration of 10^8 pfu, whereas at a fivefold higher dose, much stronger, persistent responses were observed in the majority of animals tested. This dose dependency probably accounts for the failure of a replication deficient adenovirus construct expressing rabies virus glycoprotein to elicit a measurable serum antibody response following oral immunization of mice with 10^6 pfu (Xiang *et al.,* 1996). We have previously reported a strong dose-dependent effect following systemic immunization of mice with an HIV-1 *env/rev*-expressing construct; however, in this instance a single immunization induced CTL memory in the absence of an antibody response (Bruce *et al.,* 1999). A similar finding was reported by Flanagan *et al.* (1997) following oral administration of an SIV Gag p55-expressing replication competent construct. The MV N protein may be a more potent B-cell immunogen, as the protein can self assemble into nucleocapsid-like structures within RAd68-infected cells (Fooks *et al.,* 1995). Virus and virus-like particles expressing repeated B-cell epitopes are known to efficiently induce T-cell-independent antibody responses (Fehr *et al.,* 1998) and persistence of immune complexed antigen may account for the longevity of the antibody response observed (Bachmann *et al.,* 1994). Long-term antibody responses may be maintained by the ongoing differentiation of memory B cells into antibody-secreting plasma cells. However, it has also been shown that plasma cells themselves may be long-lived (Manz *et al.,* 1997; Slifka *et al.,* 1998) and can survive independently of antigen (Manz *et al.,* 1999). Likewise, longevity of the N protein-specific CTL memory responses may also either be due to persistence of antigen, although even with replication-competent constructs expression may be relatively short-lived (Mittal *et al.,* 1993), or memory pCTL may be maintained in the absence of specific or cross-reactive antigenic stimulation (Murali-Krishna *et al.,* 1999). Interestingly, the lower dose of RAd68 induced a stronger response at higher frequency in mesenteric lymph nodes shortly after infection than did the higher dose, often in the absence of a splenic response. Surprisingly, by week 4 mesenteric responses were undetectable in animals given the higher dose of virus despite the efficient generation of splenic responses. The generation of long-lived splenic memory CTL responses following oral delivery differs from the findings reported following intranasal delivery of a replication competent adenovirus expressing herpes simplex glycoprotein B. In that study, memory CTL responses were detected locally and systemically at early times, as in the present study; however, after several months a local memory response persisted in the absence of a splenic memory response (Gallichan and Rosenthal, 1996). Our results suggest that the magnitude of the dose given orally may determine the distribution of the CTL memory response. Replication deficient adenoviruses can transduce enterocytes and can express transgenes at a high level in epithelial cells, possibly M cells, overlying lymphoid follicle domes in the distal ileum (Foreman *et al.,* 1998). Thus, persistence of expression at these sites may efficiently stimulate immune responses. The apparent disappearance of local reactivity may be due to the presence of effector cells stimulated *in vivo* that would undergo apoptosis under conditions of *in vitro* restimulation. In future studies this possibility could be addressed by analysis of CD8 cell reactivity directly *ex vivo* using sensitive assays such as Elispot, intracellular cytokine staining, or staining with MHC–peptide tetrameric complexes. We cannot exclude the possibility that at high doses of virus there is a degree of breakthrough of virus replication. High multiplicity infection *in vitro* with RAd68 leads to expression of adenovirus structural proteins (A. Fooks, unpublished observation). However, given the size of the target cell population in the gut, it would seem unlikely that such conditions would occur *in vivo.* Further studies using *in situ* labeling techniques and virus rescue are needed to fully address these issues of antigen persistence and localized virus replication in this model system.

Our results show that the oral (intragastric) route is efficient for the stimulation of persistent immune responses to a transgene expressed from a replication deficient adenovirus following a single administration and may stimulate systemic and local responses in a dose-dependent manner. In this study we did not analyze local antibody responses; however, we believe that our results are sufficiently encouraging to stimulate further studies in this area. Oral delivery of DNA by this vector is much more efficient than administration of either naked DNA (Etchart *et al.,* 1997) or DNA, encoding the same antigen, encapsulated in biodegradable microspheres (Fooks *et al.,* 2000). In addition to the potential of this vaccination strategy as a complementary approach in

the eradication of measles virus, oral/gastric delivery of recombinant replication deficient adenovirus constructs in a single dose has great potential in vaccination against many infections acquired via mucosal surfaces including HIV.

MATERIALS AND METHODS

Cells and viruses

The construction of the adenovirus recombinant (RAd68) expressing the MV nucleocapsid protein has been described previously (Fooks *et al.,* 1995). Briefly, the intact N gene of the Edmonston-P9 strain of MV was placed under the control of the HCMV IE promoter and recombined into the E1 region of human adenovirus 5 vector. Stocks of RAd68 were prepared following plaque purification on 293 cells that provide E1a *in trans,* thus allowing production of progeny virus. Titers were expressed as pfu determined on 293 cells.

L929 cells (H-2^k), used as targets in CTL assays, were grown as monolayers in complete Glasgow modified Eagle's medium supplemented with 10% (v/v) fetal calf serum and 0.3% (v/v) L-glutamine.

Immunization of mice

Animals were housed in accordance with the Home Office Code of Practice (1984) and licensed procedures carried out in accordance with the Animals (Scientific Procedures) Act 1986. Adult, 6- to 8-week-old C3H/He mice (Harlan-Olac) were immunized with RAd68 using a single dose containing 10^8 pfu (70 mice) or 5×10^8 pfu (70 mice). For oral immunization, food was withheld for 6 h before administration of virus (diluted to a final volume of 500 μl with 0.1 M bicarbonate buffer, pH 8.9) by gavage using a stainless-steel 22-gauge oral dosing cannula. Control groups of mice were immunized intraperitoneally with 10^8 pfu RAd68 in 100 μl of phosphate-buffered saline. Each mouse was individually earmarked for identification purposes prior to immunization. Groups of 10 mice from each immunization schedule were humanely sacrificed at weeks 2, 4, 8, 13, 26, 36, and 51 postvaccination and spleens were removed for use in the CTL assay. In addition, mesenteric lymph nodes were removed from animals sacrificed at weeks 2, 4, and 8 for CTL analysis. Tail bleeds were taken for serum antibody analysis from all of the mice remaining in the study at the same time points.

Serological analysis

IgG responses specific for MV N protein were analyzed by ELISA using baculovirus-expressed N protein as antigen as described previously (Warnes *et al.,* 1994). End-point titers were expressed as the reciprocal of the dilution of serum corresponding to a defined absorbancy on the linear region of the dose–response curve.

Analysis of CTL responses

CTL activity was assessed following expansion *in vitro* of specific precursors (pCTL) using the MV-N H-$2K^k$ restricted CTL epitope (NP9) VESPGQLI (Beauverger *et al.,* 1994). Alongside the orally immunized animals, CTL activity was also assessed at each time point in two mice immunized intraperitoneally 2 weeks earlier with 10^8 pfu RAd68 and in two naive animals (positive and negative assay controls). Splenocytes (at every time point) and lymphocytes from mesenteric lymph nodes (at weeks 2, 4, and 8) were isolated from individual mice and expanded separately. Splenocytes were cultured in 10 ml and mesenteric lymphocytes in 1 ml of lymphocyte medium (RPMI 1640 supplemented with 10% fetal calf serum, penicillin/streptomycin 100 U/ml, 20 mM HEPES, and 15 mM β-mercaptoethanol) in the presence of the NP9 peptide at 5 μg/ml. On day 3, cultures were supplemented with Lymphocult T (Biotest, UK) at 10 IU/ml and CTL activity was assessed on day 7 by chromium release assay.

On the day of assay, effector cells were washed three times, suspended in lymphocyte medium and in triplicates (splenocytes) or duplicates (mesenteric lymphocytes), diluted twofold in U-bottom wells (96-well plate; Nunc) to give effector to target ratios of 100:1, 50:1, and 25:1 for splenocytes and between 13:1 and 90:1 for mesenteric lymphocytes depending upon recovery of viable cells. Five thousand ^{51}Cr-labeled, peptide-pulsed L929 target cells were added to the effector cells in lymphocyte medium and incubated at 37°C for 5 h. Effector cells were also combined with targets in the absence of peptide to determine the no-peptide background percentage lysis. Spontaneous and total chromium releases were estimated from wells in which target cells were incubated in medium alone or with 5% Triton X-100. The percentage specific lysis was determined as (sample release − spontaneous release)/(total release − spontaneous release) × 100. Only responses that exceeded the background level of killing by 2 standard deviations above the mean result from 18 naive mice (splenocytes) or from 6 naive mice (mesenteric lymphocytes) were scored as positive.

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